(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 January 2001 (11.01.2001)

PCT

(10) International Publication Number
WO 01/02597 A1

(51) International Patent Classification[7]: C12P 19/26, C12N 1/20, C08B 37/00

(21) International Application Number: PCT/EP00/06122

(22) International Filing Date: 30 June 2000 (30.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
MI99A001465 2 July 1999 (02.07.1999) IT

(71) Applicant *(for all designated States except US)*: **INALCO S.P.A.** [IT/IT]; Via Calabiana, 18, I-20139 Milan (IT).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **PETRUCCI, Franco** [IT/IT]; Residenza Mestieri, Milano 2, I-20090 Segrate (IT). **ZOPPETTI, Giorgio** [IT/IT]; Via Mac Mahon 43, I-20155 Milano (IT). **ORESTE, Pasqua** [IT/IT]; Via Mac Mahon 43, I-20155 Milano (IT). **CIPOLLETTI, Giovanni** [IT/IT]; Via Tagliamento, 21, I-20139 Milano (IT).

(74) Agent: **GERVASI, Gemma**; Notarbartolo & Gervasi S.p.A., Corso di Porta Vittoria, 9, I-20122 Milano (IT).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PROCESS FOR THE PREPARATION OF THE POLYSACCHARIDES K4 AND K5 FROM *ESCHERICHIA COLI*

(57) Abstract: Process for the preparation of the polysaccharides K4 and K5 comprising the fermentation in a submerged culture of a strain of *Escherichia coli* which produces the polysaccharide K4 or of a strain of *Escherichia coli* which produces the polysaccharide K5, in which the culture medium for said fermentation is composed of defatted soya flour, mineral salts and glucose, or of the dialysed portion of yeast autolysate, mineral salts and glucose.

WO 01/02597 A1

PROCESS FOR THE PREPARATION OF THE POLYSACCHARIDES K4 AND K5 FROM *ESCHERICHIA COLI*

Field of the Invention

The present invention refers to the preparation of the polysaccharides K4 and K5 from *Escherichia coli* carried out by a fermentation process.

Prior Art

The polysaccharides K4 and K5 are known which may be obtained from strains of *Escherichia coli* responsible for extra-intestinal infections.

The polysaccharide K4 is composed of equimolecular quantities of glucuronic acid and N-acetylgalactosamine in a linear chain bonded with β-1,3 bonds and fructose bonded in a lateral chain with carbon 3 of glucuronic acid.

The polysaccharide K5 is composed of equimolecular quantities of glucuronic acid and N-acetylglucosamine, which make up the alternate linear repetitive unit 4-β-glucuronil-1,4 α-N-acetylglucosamine. This repetitive unit presents a structural analogy with completely desulphated and N-acetylated heparin, hence the use of K5 may be hypothesised in processes of semi-synthesis to obtain heparin-like sulphated polysaccharides. The possibility of producing the polysaccharides K4 and K5 by means of a fermentative process therefore opens up new prospects for obtaining biologically active molecules by semi-synthesis as an alternative to extraction from animal organs.

The polysaccharide K5 was initially obtained in capsular form from broth culture *in toto* with yields of about 40-50 mg/l of broth culture [Eur. J. Biochem. 116, 359-369, 1981].

Subsequently the production of the polysaccharide K5 in extracellular form was described, which contemplates the isolation of K5 from the culture filtrate with yields varying from 200 to 700 mg/l of broth culture [J. Bioact. Comp. Polym. 11, 301-311, 1996; European Patent EP 0489 647 A2, 1991].

Also the polysaccharide K4 was initially obtained in capsular form from broth culture *in toto* with yields of about 80-90 mg/l of broth culture [Eur. J. Biochem. 117, 112-124, 1988].

Subsequently the production of the polysaccharide K4 in extracellular form was described, which contemplates the isolation of K4 from the culture filtrate alone

with yields of about 200 mg/l of broth culture [Biotechnol. Letters 18, 383-386, 1996].

The yields given above do not allow economically advantageous results.

Summary

We have now found a new process which allows the polysaccharides K4 and K5 to be obtained by fermentation, isolating them from broth cultures of *Escherichia coli*".

The process for the production, isolation and purification of the polysaccharides K4 and K5 according to this invention allows these polysaccharides to be obtained with high purity and with higher yields than when using the known processes.

The invention process comprises the following stages:

a) fermentation in a submerged culture of a strain of *Escherichia coli* which produces the polysaccharide K4 or of a strain of *Escherichia coli* which produces the polysaccharide K5;

b) centrifugation of the broth culture, concentration of the culture filtrate by ultrafiltration and precipitation of the polysaccharide by treatment with an organic solvent;

c) dissolving of the precipitate in a suitable buffer solution and treatment with protease;

d) passage through an ion exchange column followed by one or more stages of dialysis and re-precipitation with ethanol.

The innovation of the process lies in the fact that the culture medium for said fermentation is an aqueous medium comprising: defatted soya flour, mineral salts and glucose, or the dialysed portion of yeast autolysate, mineral salts and glucose.

Detailed description of the invention

The characteristics and the advantages of the process for the preparation of the polysaccharides K4 and K5 by fermentation, isolating them from broth cultures of *Escherichia coli*" according to this invention, will be illustrated at greater length during the following description. The strains of *Escherichia coli* suitable for the production of K4 or of K5 may be obtained from public collections such as the International *Escherichia* Centre [Denmark], ATCC [American Type Culture Collection – USA], DSM [Deutsche Sammlung von Mikroorganismen, Federal

Republic of Germany] and others. Alternatively, strains of *E. coli* which produce K4 or K5 may be obtained by isolation from clinical findings and subsequent characterisation as described in the literature [Eur. J. Biochem. 117, 112-124, 1988].

In the experimentation for this invention, the following strains were used:
E. coli O5:K4:H4 available from ATCC number 23502, for the preparation of K4;
E. coli O10:K5:H4 available from ATCC number 23506, for the preparation of K5.
These strains present the following characteristics (+ = positive and - = negative).

Reaction/Enzyme	Substrate	E.coli O10:K5:H4	E.coli O5:K4:H4
β-galactosidase	O-nitrophenyl-galactoside	+	+
arginine dehydrolase	Arginine	-	-
lysine decarboxylase	Lysine	+	+
ornithine decarboxylase	Ornithine	-	-
use of citrate	Sodium citrate	-	-
production of H_2S	Sodium thiosulphate	-	-
urease	Urea	-	-
tryptophan deaminase	Tryptophan	-	-
production of indol	Tryptophan	+	+
production of acetoine	Sodium pyruvate	-	-
gelatinase	Gelatine	-	-
fermentation of glucose	Glucose	+	+
fermentation-oxidation (other S)	Mannitol	+	+
fermentation-oxidation	Inositol	-	-
fermentation-oxidation	Sorbitol	+	+
fermentation-oxidation	Rhamnose	+	+
fermentation-oxidation	Saccharose	-	-
fermentation-oxidation	Melibiose	+	+
fermentation-oxidation	Amygdalin	-	-

fermentation-oxidation	Arabinose	+	+
cytochrome oxidase	Tetramethylpara enyldiamine	-	-
production of NO_2	Glucose	+	+
reduction from NO_2 to N_2	Glucose	+	+

The fermentation conditions in submerged culture may vary according to the characteristics of the strain used. Proceeding according to this invention, the fermentation temperature is between 30 and 40°C, and preferably 37°C, while the time is between 2 and 24 hours. As regards the culture medium we found that the fermentation yield of K4 and of K5 in the extracellular form is particularly high when using as a culture medium an aqueous medium with the ingredients defatted soya flour (0.1-5 g/l) or the dialysed portion of yeast autolysate (from 5 to 30 g/l) (10 g are dissolved in 50 ml and dialysed against 500 ml of water), mineral salts and glucose.

The content of said mineral salts and glucose is as follows: from 5 to 15 g/l of K_2HPO_4, from 0.5 to 5 g/l of KH_2PO_4, from 0.01 to 1 g/l of $MgCl_2$, from 0.05 to 2 g/l of sodium citrate, from 0.1 to 3 g/l of ammonium sulphate and from 0.5 to 4 g/l of glucose.

At the end of fermentation the cells are separated from the culture mixture, preferably by centrifugation. The culture filtrate is then concentrated to 1/4 - 1/5 of the initial volume, for example by ultrafiltration with a tangential flow or using flat or spiralled membranes with a different molecular cut-off (8,000-300,000 D).

The precipitation of the polysaccharide K4 or K5 is carried out at 4°C overnight, using 4-5 volumes of solvent (96% ethanol or isopropanol or acetone) per volume of concentrated culture filtrate. The precipitate collected for example by centrifugation, dissolved in a suitable buffer, is subjected to enzymatic deproteinization, using a fungal protease (for example Protease Type XXIII: fungal crude from *Aspergillus oryzae*).

The subsequent purification of the polysaccharide is carried out by passing it through an ion exchange column followed by one or more stages of dialysis, for example for ultrafiltration against distilled water, and subsequent re-precipitations

with ethanol. Normally two cycles of dialysis and precipitations are sufficient to obtain the polysaccharide in a sufficiently pure form. The sample may later by dehydrated using the traditional techniques such lyophilization, treatment with solvents and drying.

For the purpose of illustration, the following examples are given which describe in detail the process of the invention for the production of the polysaccharides K4 and K5.

Example 1

Preparation of the polysaccharide K5 in a flask with soya flour.

For the production of the polysaccharide K5 in a flask, 750 ml flasks with a deflector were used, each containing 100 ml of culture medium.

Defatted soya flour was used (PROVABIS Prodotti Gianni, Milan) (2 g/2) and the culture medium SD having the following composition (g/l):

K_2HPO_4: 9.7

KH_2PO_4: 2.0

$MgCl_2$: 0.1

sodium citrate: 0.5

ammonium sulphate: 1.0

glucose: 2.0

well water: 1,000 ml, pH 7.3, sterilisation at 118°C for 20 min.

The glucose solution was prepared separately, sterilised at 115°C for 30 min. and added in sterile condition to the culture medium.

Each flask was inoculated starting from a cellular suspension from a slant of TSA (Tryptic Soya Agar) incubated at 37°C for 24 h. The flasks were placed to incubate with alternative agitator (6 cm of movement, 160 cpm) at 37°C for 24 h and samples were taken at different times. The microbe development was assessed by means of a direct count in the optical microscope using the Bürker chamber.

At the end of fermentation, heat treatment was carried out (80°C for 10 min) in order to inactivate any enzymatic components present.

Isolation of the polysaccharide K5

The broth culture obtained by means of the described fermentation was

centrifuged at 10,000 rpm in order to separate the cells from the culture filtrate.

The following operations were performed to isolate the polysaccharide from the culture filtrate:

Concentration

The culture filtrate (1,000 ml) was subjected to concentration by ultrafiltration using membranes with cut-off 8,000-10,000 D down to a final volume of about 1/5 of the initial volume (200 ml). During this phase various types of filtering modules may be used; in particular, in this preparation a *Minitan* cell (Millipore) was used with flat membranes of polysulfone (PTCG).

Precipitation

The polysaccharide K5 was precipitated by adding 4 volumes (800 ml) of 96% ethanol at 4°C overnight. The polysaccharide K5 has a natural tendency to sediment, thus making it possible to separate most of the supernatant liquor by siphoning; the residual precipitate was then separated by centrifugation at 10,000 rpm for 20 min. These conditions were adopted in all the subsequent precipitation stages.

Deproteinization

The precipitate was subjected to enzymatic deproteinization, using a fungal protease (Protease Type XXIII: fungal crude from *Aspergillus oryzae* 3.2 U/mg, code 4755, Sigma) adopting the following conditions:

- the precipitate was dissolved in 45 ml of a solution of NaCl 0.1 M-EDTA 0.15 M at pH 8, containing sodium dodecylsulphate (SDS) at 0.5% (p/v);
- addition of 4 U of protease per litre of initial culture filtrate and thermostat control at 37°C for 90 min:
- dialysis by ultrafiltration (2 cycles) using the *Minitan* cell, with 50 ml of distilled water;
- precipitation with ethanol.

Purification

Purification was carried out by means of two extraction cycles with 30 ml of NaCl 1 M, followed by dialysis by ultrafiltration with 50 ml of distilled water and precipitation with ethanol.

The precipitate obtained from the second precipitation was dissolved in H_2O and

subjected to chromatography with ion exchange on a DEAE column, washing the column with 1 volume of NaCl 0.4 M and separating the polysaccharide with 1 volume of NaCl 0.6 M.

The fermentation yield in purified K5 is about 850 mg/l.

Characteristics of K5

The samples obtained at the end of purification were analysed according to the following procedures and with the following results.

Content of uronic acids about 40% determined using the carbazole method (Bitter *et al.*, Anal. Biochem. 4, 330-334, 1962).

Spectrometry ^{13}C NMR:

- Varian Gemini 200 operating at 50.3 MHz at 22°C using TSP as standard;
- sample 50 mg/ml in D_2O.

The spectrums of the polysaccharide produced were similar to the spectrums for the polysaccharide K5 reported in the literature.

The principal signals related to N-acetylglucosamine and to D-glucuronic acid bonded in sequence were particularly shown.

Assessment of the molecular weight by HPLC chromatography with molecular exclusion:

column: (1 x 30 cm) Superdex 75 HR (Pharmacia)

eluent: phosphate buffer 0.1 M, pH 7 with addition of NaCl 0.15 M

flow: 1 ml/min

detector: UV at 210 nm

sample: 20 l of a solution 2 mg/ml.

The polysaccharide was made up of two components: one with an apparent PM of about 16,000 D (70%) and the other with PM 5,000 D (30%).

Example 2

Preparation of the polysaccharide K5 in a flask with soya flour.

The polysaccharide K5 was prepared as in example 1 using the culture medium SD and 2 g/l of defatted soya flour (Cargill Foods S.R.L., Milan, 200/20 S).

At the end of fermentation, heat treatment was performed as described in example 1.

The polysaccharide was separated with the same procedure used in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out in the same conditions as example 1.

The fermentation yield in purified K5 is about 800 mg/l.

The samples of K5 obtained presented a content of uronic acids, dosed with the carbazole method, of about 40%.

The polysaccharide was made up of two components: one with an apparent PM of about 16,000 D (70-80%) and the other with PM 5,000 D (20-30%).

Example 3

Preparation of the polysaccharide K5 in a flask with soya flour.

The polysaccharide K5 was prepared as in example 1 using the culture medium SD and 2 g/l of defatted soya flour (Cargill Foods S.R.L., Milan, 100/20 S).

At the end of fermentation, heat treatment was performed as described in example 1.

The polysaccharide was separated with the same procedure used in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out in the same conditions as example 1.

The fermentation yield in purified K5 is about 900 mg/l.

The samples of K5 obtained presented a content of uronic acids, dosed with the

carbazole method, of about 40%.

The polysaccharide was made up of two components: one with an apparent PM of about 16,000 D (70-80%) and the other with PM 5,000 D (20-30%).

Example 4

Preparation of the polysaccharide K5 in a flask with soya flour.

The polysaccharide K5 was prepared as in example 1 using the culture medium SD and 2 g/l of defatted soya flour (Cargill Foods S.R.L., Milan, 200/20 S).

At the end of fermentation, heat treatment was performed as described in example 1.

The polysaccharide was separated with the same procedure used in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out in the same conditions as example 1.

The fermentation yield in purified K5 is about 780 mg/l.

The samples of K5 obtained presented a content of uronic acids, dosed with the carbazole method, of about 40%.

The polysaccharide was made up of two components: one with an apparent PM of about 16,000 D (70-80%) and the other with PM 5,000 D (20-30%).

Example 5

Preparation of the polysaccharide K5 in a flask with soya flour.

The polysaccharide K5 was prepared as in example 1 using the culture medium SD and 2 g/l of defatted soya flour (ABS Food - Defatted Soya Flour – India).

At the end of fermentation, heat treatment was performed as described in example 1.

The polysaccharide was separated with the same procedure used in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out in the same conditions as example 1.

The fermentation yield in purified K5 is about 820 mg/l.

The samples of K5 obtained presented a content of uronic acids, dosed with the carbazole method, of about 40%.

The polysaccharide was made up of two components: one with an apparent PM of about 16,000 D (70-80%) and the other with PM 5,000 D (20-30%).

Example 6

Preparation of the polysaccharide K5 in a flask with a proteinaceous concentrate extracted from soya flour.

The polysaccharide K5 was prepared as in example 1 using the culture medium SD and 2 g/l of proteinaceous concentrate obtained from defatted soya flour (ABS Food – ABSPS60 – India).

At the end of fermentation, heat treatment was performed as described in example 1.

The polysaccharide was separated with the same procedure used in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out in the same conditions as example 1.

The fermentation yield in purified K5 is about 720 mg/l.

The samples of K5 obtained presented a content of uronic acids, dosed with the carbazole method, of about 40%.

The polysaccharide was made up of two components: one with an apparent PM of about 16,000 D (70-80%) and the other with PM 5,000 D (20-30%).

Example 7

Preparation of the polysaccharide K5 in a flask with a proteinaceous concentrate extracted from soya flour.

The polysaccharide K5 was prepared as in example 1 using the culture medium SD and 2 g/l of defatted soya flour (Cereol).

At the end of fermentation, heat treatment was performed as described in example 1.

The polysaccharide was separated with the same procedure used in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out in the same conditions as example 1.

The fermentation yield in purified K5 is about 710 mg/l.

The samples of K5 obtained presented a content of uronic acids, dosed with the carbazole method, of about 40%.

The polysaccharide was made up of two components: one with an apparent PM of about 16,000 D (70-80%) and the other with PM 5,000 D (20-30%).

Example 8

Preparation of the polysaccharide K5 in a flask with soya flour.

The polysaccharide K5 was prepared as in example 1 using the culture medium SD and 1 g/l of defatted soya flour (Cargill Foods S.R.L., Milan, 100/20 S).

At the end of fermentation, heat treatment was performed as described in example 1.

The polysaccharide was separated with the same procedure used in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out in the same conditions as example 1.

The fermentation yield in purified K5 is about 780 mg/l.

The samples of K5 obtained presented a content of uronic acids, dosed with the carbazole method, of about 40%.

The polysaccharide was made up of two components: one with an apparent PM of about 16,000 D (70-80%) and the other with PM 5,000 D (20-30%).

Example 9

Preparation of the polysaccharide K5 in a flask with soya flour.

The polysaccharide K5 was prepared as in example 1 using the culture medium SD and 1 g/l of defatted soya flour (ABS Food - Defatted Soya Flour – India).

At the end of fermentation, heat treatment was performed as described in example 1.

The polysaccharide was separated with the same procedure used in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out in the same conditions as example 1.

The fermentation yield in purified K5 is about 815 mg/l.

The samples of K5 obtained presented a content of uronic acids, dosed with the carbazole method, of about 40%.

The polysaccharide was made up of two components: one with an apparent PM of about 16,000 D (70-80%) and the other with PM 5,000 D (20-30%).

Example 10

Preparation of the polysaccharide K5 in a flask with soya flour.

The polysaccharide K5 was prepared using the culture medium SD as in example 1 and 1 g/l of defatted soya flour (Cargill Foods S.R.L., Milan, 100/20 S).

Each flask was inoculated with 1% (v/v) using a culture in liquid of 16 h prepared in the same culture medium.

At the end of fermentation, heat treatment was performed as described in example 1.

The polysaccharide was separated with the same procedure used in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out in the same conditions as example 1.

The fermentation yield in purified K5 is about 750 mg/l.

The samples of K5 obtained presented a content of uronic acids, dosed with the carbazole method, of about 40%.

The polysaccharide was made up of two components: one with an apparent PM of about 16,000 D (70-80%) and the other with PM 5,000 D (20-30%).

Example 11

Preparation of the polysaccharide K5 in a flask with soya flour.

The polysaccharide K5 was prepared using the culture medium SD as in example 1 and 1 g/l of defatted soya flour (Cargill Foods S.R.L., Milan, 100/20 S).

Each flask was inoculated with 10% (v/v) using a culture in liquid of 16 h prepared

in the same culture medium.

At the end of fermentation, heat treatment was performed as described in example 1.

The polysaccharide was separated with the same procedure used in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out in the same conditions as example 1.

The fermentation yield in purified K5 is about 830 mg/l.

The samples of K5 obtained presented a content of uronic acids, dosed with the carbazole method, of about 40%.

The polysaccharide was made up of two components: one with an apparent PM of about 16,000 D (70-80%) and the other with PM 5,000 D (20-30%).

Example 12

Preparation of the polysaccharide K5 in a flask with soya flour.

The polysaccharide K5 was prepared using the culture medium SD as in example 1 and 2 g/l of defatted soya flour (Cargill Foods S.R.L., Milan, 100/20 S).

Each flask was inoculated with 1% (v/v) using a culture in liquid of 16 h prepared in the same culture medium.

At the end of fermentation, heat treatment was performed as described in example 1.

The polysaccharide was separated with the same procedure used in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out in the same conditions as example 1.

The fermentation yield in purified K5 is about 800 mg/l.

The samples of K5 obtained presented a content of uronic acids, dosed with the carbazole method, of about 40%.

The polysaccharide was made up of two components: one with an apparent PM of about 16,000 D (70-80%) and the other with PM 5,000 D (20-30%).

Example 13

Preparation of the polysaccharide K5 in a flask with soya flour.

The polysaccharide K5 was prepared using the culture medium SD as in example 1 and 2 g/l of defatted soya flour (Cargill Foods S.R.L., Milan, 100/20 S).

Each flask was inoculated with 10% (v/v) using a culture in liquid of 16 h prepared in the same culture medium.

At the end of fermentation, heat treatment was performed as described in example 1.

The polysaccharide was separated with the same procedure used in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out in the same conditions as example 1.

The fermentation yield in purified K5 is about 930 mg/l.

The samples of K5 obtained presented a content of uronic acids, dosed with the carbazole method, of about 40%.

The polysaccharide was made up of two components: one with an apparent PM of about 16,000 D (70-80%) and the other with PM 5,000 D (20-30%).

Example 14

Preparation of the polysaccharide K5 in a flask with soya flour.

The polysaccharide K5 was prepared using the culture medium SD as in example 1 and 1 g/l of defatted soya flour (ABS Food - Defatted Soya Flour – India).

Each flask was inoculated with 1% (v/v) using a culture in liquid of 16 h prepared in the same culture medium.

At the end of fermentation, heat treatment was performed as described in example 1.

The polysaccharide was separated with the same procedure used in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out in the same conditions as example 1.

The fermentation yield in purified K5 is about 790 mg/l.

The samples of K5 obtained presented a content of uronic acids, dosed with the carbazole method, of about 40%.

The polysaccharide was made up of two components: one with an apparent PM of about 16,000 D (70-80%) and the other with PM 5,000 D (20-30%).

Example 15

Preparation of the polysaccharide K5 in a flask with soya flour.

The polysaccharide K5 was prepared using the culture medium SD as in example 1 and 1 g/l of defatted soya flour (ABS Food - Defatted Soya Flour – India).

Each flask was inoculated with 10% (v/v) using a culture in liquid of 16 h prepared in the same culture medium.

At the end of fermentation, heat treatment was performed as described in example 1.

The polysaccharide was separated with the same procedure used in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out in the same conditions as example 1.

The fermentation yield in purified K5 is about 810 mg/l.

The samples of K5 obtained presented a content of uronic acids, dosed with the carbazole method, of about 40%.

The polysaccharide was made up of two components: one with an apparent PM of about 16,000 D (70-80%) and the other with PM 5,000 D (20-30%).

Example 16

Preparation of the polysaccharide K5 in a flask with soya flour.

The polysaccharide K5 was prepared using the culture medium SD as in example 1 and 2 g/l of defatted soya flour (ABS Food - Defatted Soya Flour – India).

Each flask was inoculated with 1% (v/v) using a culture in liquid of 16 h prepared in the same culture medium.

At the end of fermentation, heat treatment was performed as described in example 1.

The polysaccharide was separated with the same procedure used in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out in the same conditions as example 1.

The fermentation yield in purified K5 is about 810 mg/l.

The samples of K5 obtained presented a content of uronic acids, dosed with the carbazole method, of about 40%.

The polysaccharide was made up of two components: one with an apparent PM of about 16,000 D (70-80%) and the other with PM 5,000 D (20-30%).

Example 17

Preparation of the polysaccharide K5 in a flask with soya flour.

The polysaccharide K5 was prepared using the culture medium SD as in example 1 and 2 g/l of defatted soya flour (ABS Food - Defatted Soya Flour – India).

Each flask was inoculated with 10% (v/v) using a culture in liquid of 16 h prepared in the same culture medium.

At the end of fermentation, heat treatment was performed as described in example 1.

The polysaccharide was separated with the same procedure used in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out in the same conditions as example 1.

The fermentation yield in purified K5 is about 820 mg/l.

The samples of K5 obtained presented a content of uronic acids, dosed with the carbazole method, of about 40%.

The polysaccharide was made up of two components: one with an apparent PM of about 16,000 D (70-80%) and the other with PM 5,000 D (20-30%).

Example 18

Preparation of the polysaccharide K5 in a flask with soya flour.

The polysaccharide K5 was prepared using the culture medium SD as in example 1 and 1 g/l of defatted soya flour (Cargill Foods S.R.L., Milan, 100/20 S).

Each flask was inoculated with 10% (v/v) using a culture in liquid of 16 h prepared in the same culture medium and prolonging the fermentation time to 30 h.

At the end of fermentation, heat treatment was performed as described in example 1.

The polysaccharide was separated with the same procedure used in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out in the same conditions as example 1.

The fermentation yield in purified K5 is about 850 mg/l.

The samples of K5 obtained presented a content of uronic acids, dosed with the carbazole method, of about 40%.

The polysaccharide was made up of two components: one with an apparent PM of about 16,000 D (70-80%) and the other with PM 5,000 D (20-30%).

Example 19

Preparation of the polysaccharide K5 in a flask with soya flour.

The polysaccharide K5 was prepared using the culture medium SD as in example 1 and 1 g/l of defatted soya flour (Cargill Foods S.R.L., Milan, 100/20 S).

Each flask was inoculated with 10% (v/v) using a culture in liquid of 16 h prepared in the same culture medium and prolonging the fermentation time to 48 h.

At the end of fermentation, heat treatment was performed as described in example 1.

The polysaccharide was separated with the same procedure used in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as

example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out in the same conditions as example 1.

The fermentation yield in purified K5 is about 710 mg/l.

The samples of K5 obtained presented a content of uronic acids, dosed with the carbazole method, of about 40%.

The polysaccharide was made up of two components: one with an apparent PM of about 16,000 D (70-80%) and the other with PM 5,000 D (20-30%).

Example 20

Preparation of the polysaccharide K5 in a flask with soya flour.

The polysaccharide K5 was prepared using the culture medium SD as in example 1 and 2 g/l of defatted soya flour (Cargill Foods S.R.L., Milan, 100/20 S).

Each flask was inoculated with 10% (v/v) using a culture in liquid of 16 h prepared in the same culture medium and prolonging the fermentation time to 30 h.

At the end of fermentation, heat treatment was performed as described in example 1.

The polysaccharide was separated with the same procedure used in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out in the same conditions as example 1.

The fermentation yield in purified K5 is about 830 mg/l.

The samples of K5 obtained presented a content of uronic acids, dosed with the carbazole method, of about 40%.

The polysaccharide was made up of two components: one with an apparent PM of

about 16,000 D (70-80%) and the other with PM 5,000 D (20-30%).

Example 21

Preparation of the polysaccharide K5 in a flask with soya flour.

The polysaccharide K5 was prepared using the culture medium SD as in example 1 and 2 g/l of defatted soya flour (Cargill Foods S.R.L., Milan, 100/20 S).

Each flask was inoculated with 10% (v/v) using a culture in liquid of 16 h prepared in the same culture medium and prolonging the fermentation time to 48 h.

At the end of fermentation, heat treatment was performed as described in example 1.

The polysaccharide was separated with the same procedure used in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out in the same conditions as example 1.

The fermentation yield in purified K5 is about 720 mg/l.

The samples of K5 obtained presented a content of uronic acids, dosed with the carbazole method, of about 40%.

The polysaccharide was made up of two components: one with an apparent PM of about 16,000 D (70-80%) and the other with PM 5,000 D (20-30%).

Example 22

Preparation of the polysaccharide K5 in a flask with dialysed yeast autolysate.

For the production of the polysaccharide K5 in a flask, 750 ml flasks with a deflector were used, each containing 100 ml of culture medium.

The culture medium AL was used having the following composition (g/l):

yeast autolysate: 10.0 (10.0 g are dissolved in 50 ml and dialysed against 50 ml of water)

K_2HPO_4: 9.7

KH_2PO_4: 2.0

$MgCl_2$: 0.1

sodium citrate: 0.5

ammonium sulphate: 1.0

glucose: 2.0

well water: 1,000 ml, pH 7.3, sterilisation at 118°C for 20 min.

The glucose solution was prepared separately, sterilised at 115°C for 30 min. and added in sterile condition to the culture medium.

Each flask was inoculated starting from a cellular suspension from a slant of TSA (Tryptic Soya Agar) incubated at 37°C for 24 h. The flasks were placed to incubate with alternative agitator (6 cm of movement, 160 cpm) at 37°C for 24 h and samples were taken at different times. The microbe development was assessed by means of a direct count in the optical microscope using the Bürker chamber.

At the end of fermentation, heat treatment was performed as described in example 1.

The polysaccharide was separated with the same procedure used in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out in the same conditions as example 1.

The fermentation yield in purified K5 is about 780 mg/l.

The samples of K5 obtained presented a content of uronic acids, dosed with the carbazole method, of about 40%.

The polysaccharide was made up of two components: one with an apparent PM of about 16,000 D (70-80%) and the other with PM 5,000 D (20-30%).

Example 23

Preparation of the polysaccharide K5 in a fermenter.

The preparation was carried out in an automated 14 l fermenter with a working volume of 10 l (Chemap-Braun, Melsungen, Germany). The culture medium SD was used having the same composition as in example 1.

The fermentation conditions adopted were the same as in example 1, with inoculation of 10% (v/v) from a submerged culture of 24 h prepared in a flask using the same culture medium, with aeration 1 vvm (volume per volume per minute), agitation 400 rpm, temperature 37°C, fermentation time up to 24 h.

During fermentation, the following parameters were assessed:

trend of the ph

trend of the dissolved oxygen

residual glucose

polysaccharide produced

microbe development

At the end of fermentation, heat treatment was carried out (80°C for 10 min) in order to inactivate any enzymatic components present.

The polysaccharide was separated with the same procedure used in example 1.

The culture filtrate (10 l) was subjected to concentration by ultrafiltration using membranes with cut-off 8,000 and 10,000 D down to a final volume of about 2 l using a SS 316 module (MST, Gallarate, Varese) with spiralled polymer membranes (PES).

The polysaccharide was precipitated by adding 4 volumes of ethanol at 4°C overnight, then separated from the supernatant liquor by centrifugation at 10,000 rpm for 20 min.

For deproteinization the same fungal protease was used as in example 1, dissolving the precipitate in 450 ml of reaction buffer and proceeding in the same conditions as example 1, using the SS 316 module and 500 ml of distilled water for ultrafiltration.

The samples of K5 obtained at 24 h presented a content of about 70-80% of the component at 16,000 D and about 20-30% of the one at 5,000 D.

Yield: 820 mg/l.

Example 24

Preparation of the polysaccharide K4 in a flask.

The polysaccharide K4 was prepared using the medium SD in the same conditions as example 1, checking the same parameters during fermentation.

At the end of fermentation, heat treatment was carried out (80°C for 10 min) in order to inactivate any enzymatic components present.

The polysaccharide was isolated as in example 1.

Concentration

The concentration of the culture filtrate was performed as in example 1 using a 300,000 D membrane for filtration.

Precipitation

The precipitation of the polysaccharide was carried out in the same conditions as example 1.

Deproteinization

Deproteinization was carried out in the same conditions as example 1.

Purification

Purification was carried out by means of two extraction cycles with 30 ml of NaCl 1 M, followed by dialysis by ultrafiltration with 50 ml of distilled water and precipitation with ethanol.

The precipitate obtained from the second precipitation was dissolved in H_2O and subjected to chromatography with ion exchange on a DEAE column, washing the column with 1 volume of NaCl 0.4 M and separating the polysaccharide with 1 volume of NaCl 0.6 M.

The fermentation yield in purified K4 is about 400 mg/l.

Characterization of K4

The samples obtained at the end of purification were analysed according to the following procedures and with the following results.

Content of uronic acids about 25% determined using the carbazole method (Bitter *et al.*, Anal. Biochem. 4, 330-334, 1962).

Fructose content: about 14.1 % determined using the enzymatic test for fructose (hexokinase/phosphoglucoisomerase/glucose-6-phosphate dehydrogenase/NAPD Kit, Boehringer, Mannheim, Germany), after hydrolysis of the K4 (triphluoroacetic acid 0.1 M at 100°C for 30 min).

Galactosamine content: about 23% determined using the Elson-Morgan reactant after hydrolysis of the K4 (HCl 4M at 100°C for 18 h).

Spectrometry ^{13}C NMR

Varian Gemini 200 operating at 50.3 MHz at 22°C using TSP as standard.

Sample 50 mg/ml in NaCl 1 M.

The spectrums of the polysaccharide produced were similar to the spectrums for the polysaccharide K4 reported in the literature.

The principal signals related to N-acetylgalactosamine and to D-glucuronic acid bonded in sequence and to fructose bonded in a lateral chain to glucuronic acid were particularly shown.

Assessment of the molecular weight by HPLC chromatography with molecular exclusion:

column: (1 x 30 cm) Superdex 75 HR (Pharmacia)

eluent: phosphate buffer 0.1 M, pH 7 with addition of NaCl 0.15 M

flow: 1 ml/min

detector: UV at 210 nm

sample: 20 l of a solution 2 mg/ml.

The polysaccharide was made up of a single component with an apparent PM of about 300,000 D.

Example 25

Preparation of the polysaccharide K4 in a fermenter.

The preparation was carried out in an automated 14 l fermenter with a working volume of 10 l (Chemap-Braun, Melsungen, Germany).

The culture medium SD was used having the same composition as in example 1.

The following fermentation conditions were adopted: inoculation of 10% (v/v) from a submerged culture of 24 h prepared in a flask using the same culture medium as example 1, aeration 1 vvm (volume per volume per minute), agitation 400 rpm, temperature 37°C, fermentation time up to 24 h.

Isolation and purification of the polysaccharide were carried out as in example 3, using 300,000 D membranes for concentration.

Yield: 420 mg/l.

CLAIMS

1. Process for the preparation of the polysaccharides K4 and K5 comprising:

a) fermentation in a submerged culture of a strain of *Escherichia coli* which produces the polysaccharide K4 or of a strain of *Escherichia coli* which produces the polysaccharide K5;

b) centrifugation of the broth culture, concentration by means of ultrafiltration of the culture filtrate and precipitation of the polysaccharide;

c) dissolving of the precipitate and treatment with protease;

d) passage through an ion exchange column followed by dialysis and re-precipitation,

characterised in that the culture medium for said fermentation is composed of an aqueous mixture comprising defatted soya flour, mineral salts and glucose or comprising the dialysed portion of yeast autolysate, mineral salts and glucose.

2. Process as in claim 1, wherein the mineral salts are composed of K_2HPO_4, KH_2PO_4, $MgCl_2$, sodium citrate and ammonium sulphate.

3. Process as in claim 1, wherein said culture medium is composed of an aqueous mixture comprising from 0.1 to 5 g/l of defatted soya flour or from 5 to 30 g/l of the dialysed portion of yeast autolysate, from 5 to 15 g/l of K_2HPO_4, from 0.5 to 5 g/l of KH_2PO_4, from 0.01 to 1 g/l of $MgCl_2$, from 0.05 to 2 g/l of sodium citrate, from 0.1 to 3 g/l of ammonium sulphate and from 0.5 to 4 g/l of glucose.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/06122

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P19/26 C12N1/20 C08B37/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12P C08B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, MEDLINE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MANZONI MATILDE ET AL: "Extracellular K5 polysaccharide of Escherichia coli: Production and characterization." JOURNAL OF BIOACTIVE AND COMPATIBLE POLYMERS, vol. 8, no. 3, 1993, pages 251-257, XP000961226 ISSN: 0883-9115 the whole document ---	1-3
X	MANZONI M ET AL: "Culture medium influence on the Escherichia coli K5 polysaccharide production." ANNALI DI MICROBIOLOGIA ED ENZIMOLOGIA, vol. 44, no. 2, 1994, pages 207-215, XP000961225 ISSN: 0003-4649 the whole document ---	1-3

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 November 2000	13/12/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lejeune, R

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

In. .national Application No
PCT/EP 00/06122

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 333 243 A (AKZO NV) 20 September 1989 (1989-09-20) example 1	1,2
X	WO 92 17507 A (ITALFARMACO SPA ;MAX PLANCK INST FUER IMMUNBIOL (DE); TUBBY DAVID) 15 October 1992 (1992-10-15) page 21 -page 22	1-3
A	EP 0 489 647 A (SANOFI ELF) 10 June 1992 (1992-06-10) cited in the application example 1	1-3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

In...national Application No
PCT/EP 00/06122

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0333243 A	20-09-1989	AU 3090189 A	21-09-1989
		DK 115189 A	11-09-1989
		FI 891131 A	11-09-1989
		JP 1282201 A	14-11-1989
		NZ 228271 A	28-05-1991
		PT 89958 A	10-11-1989
		US 5071969 A	10-12-1991
		ZA 8901502 A	29-11-1989
WO 9217507 A	15-10-1992	GB 2254083 A	30-09-1992
		AU 1430892 A	02-11-1992
		EP 0577665 A	12-01-1994
		FI 934141 A	22-09-1993
		GB 2286193 A	09-08-1995
		HU 67208 A	28-02-1995
		IT 1254564 B	25-09-1995
		JP 7501684 T	23-02-1995
		NO 933440 A	26-10-1993
		PT 100316 A	30-07-1993
		ZA 9202313 A	02-08-1993
EP 0489647 A	10-06-1992	FR 2669932 A	05-06-1992
		AT 150033 T	15-03-1997
		AU 646112 B	10-02-1994
		AU 8836191 A	04-06-1992
		BG 60479 B	31-05-1995
		BG 95567 A	31-03-1994
		BR 9105239 A	18-08-1992
		CA 2056878 A	04-06-1992
		CS 9103668 A	16-09-1992
		DE 69125109 D	17-04-1997
		DE 69125109 T	28-08-1997
		DK 489647 T	08-09-1997
		ES 2099145 T	16-05-1997
		FI 915697 A	04-06-1992
		GR 3023541 T	29-08-1997
		HU 214986 B	28-08-1998
		IL 100229 A	04-01-1998
		JP 5001101 A	08-01-1993
		KR 214752 B	02-08-1999
		MX 9102353 A	01-06-1992
		NO 300277 B	05-05-1997
		NZ 240838 A	25-11-1993
		PL 167668 B	31-10-1995
		PT 99671 A,B	30-10-1992
		SI 9111886 A,B	28-02-1998
		RU 2099353 C	20-12-1997
		US 5550116 A	27-08-1996
		ZA 9109518 A	26-08-1992